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Promoter Cloning in the Radioresistant Bacterium *Deinococcus radiodurans*

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***Deinococcus radiodurans* is a highly radiation-resistant bacterium that is classed in a major subbranch of the bacterial domain. Since very little is known about gene expression in this bacterium, an initial study of promoters was undertaken. In order to isolate promoters and study promoter function, a series of integrative vectors for stable chromosomal insertion in *D. radiodurans* were developed. These vectors are based on *Escherichia coli* replicons that are unable to replicate autonomously in *D. radiodurans* and carry homologous sequences for replacement recombination in the *D. radiodurans* chromosome. The resulting integration vectors were used to study expression of reporter genes fused to a number of putative promoters that were amplified from the *D. radiodurans* R1 genome. Further analysis of these and other putative promoters was performed by Northern hybridization and primer extension experiments. In contrast to previous reports, the −10 and −35 regions of these promoters resembled the σ^{70} consensus sequence of *E. coli*.**

Its extraordinary tolerance to extremely high doses of ionizing radiation has made *Deinococcus radiodurans* the focus of growing scientific interest. This non-spore-forming bacterium is able to survive up to 4,000 times the lethal radiation dose for humans without mutation or loss of viability (2, 9). *D. radiodurans* is also of interest as a representative of a deeply branching family within the domain Bacteria (10). The sequence of the *D. radiodurans* R1 genome was recently published and shown to consist of two chromosomes, a megaplasmid, and one plasmid (17).

Despite the interest in *D. radiodurans*, little is known concerning basic gene expression and promoters. Earlier studies showed that *Deinococcus* promoter regions are poorly recognized in *Escherichia coli*, and *E. coli* promoters that were tested were not recognized in *D. radiodurans* (7, 14), suggesting that deinococcal promoters might be different from the classical *E. coli* σ^{70} type. However, no transcriptional analysis of deinococcal promoters has been carried out. Analysis of the recently published genome sequence revealed only three putative sigma factors, one classing with vegetative σ^{70} (*rpoD*) sequences, and two classing with extracytoplasmic alternative transcription factors (annotated as *rpoE* and DR0804 [17]). Surprisingly, orthologs of the nitrogen-starvation, general starvation, and heat shock sigma factors (*rpoN, rpoS*, and *rpoH*, respectively) were not found.

One reason for the lack of information on promoters in deinococci is the lack of convenient genetic tools for studying promoters. A promoter cloning vector has been described (7), but it involves an antibiotic resistance reporter and is a large plasmid with limited cloning sites. Therefore, we developed a suite of integrative promoter-screening vectors that allow the screening and assessment of promoter regions in *D. radiodurans* based on *lacZ* and *xylE* as reporters. These vectors were used to isolate and analyze promoter regions, and promoter regions were further defined by transcriptional analysis. Surprisingly, the −10 and −35 sequences of these promoters are similar to the *E. coli* σ^{70} sequence.

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MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1.

Chemicals and enzymes. All chemicals used were of analytical grade and, unless indicated otherwise, were obtained from Baker Chemical Co. (Phillipsburg, N.J.) or Fisher Scientific (Fair Lawn, N.J.). 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and *O*-nitrophenyl-β-D-galactopyranoside (ONPG) were from ISC Bioexpress (Kaysville, Utah) and Sigma Chemical Co. (St. Louis, Mo.), respectively. Enzymes for molecular biology were purchased from Roche Molecular Biochemicals (Indianapolis, Ind.) and New England Biolabs (Beverly, Mass.) and used according to the supplier. *Taq* and Platinum *Taq* DNA polymerases were obtained from Gibco-BRL (Gaithersburg, Md.).

Media and growth conditions. Luria-Bertani (LB) broth for growth of *E. coli* consisted of (per liter) 10 g of tryptone (Difco Laboratories, Detroit, Mich.), 5 g of yeast extract (Difco), and 10 g of NaCl (pH 7.4). TGY broth for *D. radiodurans* contained (per liter) 5 g of tryptone, 1 g of glucose, and 3 g of yeast extract (10). Solid media were prepared by addition of 1.5% agar (Difco) to either LB or TGY broth. Where necessary, media were supplemented with the appropriate antibiotics, all of which were obtained from Sigma. Ampicillin (Ap) was used at 50 μg/ml for *E. coli*. Tetracycline (Tc) was added to a final concentration of 2.5 μg/ml for *D. radiodurans*. Kanamycin (Km) was routinely used at 50 μg/ml for *E. coli* and 8 or 4 μg/ml for *D. radiodurans* grown on solid and liquid medium, respectively. Transformations of *E. coli* were performed either using commercially available cells (JM109 and TOP10 from Promega, Madison, Wis., and Invitrogen, Carlsbad, Calif., respectively) or by the $CaCl_2$ method (13). *D. radiodurans* cells were transformed as described previously (7a).

DNA manipulations. Miniscale plasmid DNA preparations of *E. coli* were obtained as described by Sambrook et al. (13). PCR products were purified using the Qiaquick PCR purification Kit (Qiagen Inc., Valencia, Calif.). Northern blot analyses were performed according to Sambrook et al. (13). PCR-generated probes were labeled for hybridization with [α-^{32}P]-dCTP (800 Ci/mmol; NEN Life Science Products, Boston, Mass.) using the Random Primed DNA labeling kit (Roche). Primers for PCR amplification, sequencing, and transcription start site mapping purposes were of varying length and were obtained from Gibco-BRL (Frederick, Md.) (Table 2). Radioactive sequencing reactions for primer extension analyses (see below) were carried out using the T7 Sequenase kit (Amersham Pharmacia Biotech, Piscataway, N.J.). Non-radioactive nucleotide sequencing was performed at the University of Washington's Department of

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype	Source or reference
E. coli		
JM109	F′ *traD36 lacI*^{q} Δ*(lacZ)M15 proA*$^+$ *B*$^+$/e14$^-$ (McrA$^-$) Δ*(lac-proAB) thi gyrA96* (Nal^r) *endA1 hsdR17*(r_k^- m_k^+) *relA1 supE44 recAl*	Promega (18)
TOP10	F$^-$ *mcrA* Δ*(mrr-hsdRMS-mcrBC)* Φ80*lacZ*Δ*M15* Δ*lacX74 recA1 deoR araD139* Δ*(ara-leu)7697. galU galK rpsL* (Str^r) *endA l nupG*	Invitrogen
GM48	F$^-$ *thr leu thi lacY galK galT ara fhuA tsx dam dcm supE44*	11
D. radiodurans R1	Wild-type strain	1
Plasmids		
pCR2.1	Cloning vector for PCR-generated products; Ap^r Km^r	Invitrogen
pCR2.1-TOPO	Like pCR2.1; uses covalently linked topoisomerase I instead of DNA ligase	Invitrogen
pUC19	General cloning vector for *E. coli*; Ap^r, 2.7 kb	18
pMTL23	General cloning vector for *E. coli*; Ap^r, 2.5 kb	3
pAY/K1	pUC19 carrying the *D. radiodurans amyE* gene disrupted by the pUC4K Km^r gene; Ap^r Km^r, 5.5 kb	This study
pAY/K2	pAY/K1 from which two *Pst*I sites were removed by partial digestion and T4 DNA polymerase treatment	This study
pROBe1	pAY/K2 carrying the *P. putida xylE* gene; Ap^r Km^r, 6.4 kb	This study
pROBe4	pAY/K2 carrying the *E. coli lacZ* gene as a PCR fragment derived from pMUTIN2mcs	This study; 7a
pAMYE4Z	pROBe4 carrying the 263-bp putative *amyE* promoter fragment amplified by PCR (primers *amyE*-190 and *amyE*82)	This study
pLEXA4Z	Like pAMYE4Z; 288-bp *lexA* promoter insert	This study
pGROES4Z	Like pAMYE4Z; 298-bp *groESL* promoter insert	This study
pPE1	pCR2.1-TOPO carrying a 713-bp *aceR* promoter fragment	This study
pPEx	Like pPE1; carrying 3′-extended promoter fragments of *amyE* (pPE2), *lexA* (pPE4), and *groESL* (pPE10)	This study
pPE11, pPE12, pPE13	pCR2.1-TOPO carrying the *polA* (479 bp), *rpoBC* (631 bp), and *rpoD* (446 bp) promoter fragments, respectively	This study

Biochemistry DNA Sequencing Facility, using an ABI Prism 377 sequencer (PE Biosystems).

Sequence comparisons and predictions. Computational analyses of DNA and predicted amino acid sequences were performed using the using the following internet-based programs. Similarity searches were carried out using the Blast algorithms available at http://www.ncbi.nlm.nih.gov/BLAST/. Amino acid sequences for these searches were retrieved from the Colibri (http://bioweb.pasteur.fr/GenoList/Colibri/) and SubtiList (http://bioweb.pasteur.fr/GenoList/SubtiList/) webservers, dedicated to the *E. coli* and *Bacillus subtilis* genome sequences, respectively. Multiple alignments were performed using ClustalW (http://www2.ebi.ac.uk/clustalw/). The presence of possible signal peptidase I cleavage sites was analyzed using the parameters at http://www.cbs.dtu.dk/services/SignalP/; analysis of primary protein structure was performed using the ExPASy ProtParam tool available at http://www.expasy.ch/cgi-bin/protparam. Preliminary sequence data for *D. radiodurans* were obtained from the Institute for Genomic Research website at http://www.tigr.org.

Plasmid constructions. The pAY/K and pROBe series of promoter probe vectors were constructed as follows. First, a PCR fragment carrying the putative *D. radiodurans* R1 α-amylase (1,4-α-D-glucan glucanohydrolase) gene (*amyE*) was cloned in pCR2.1 (Invitrogen). The *amyE* gene was subsequently transferred to the *Eco*RI site of pUC19 and disrupted by insertion of the pUC4K Km marker in the *Hin*cII site, yielding pAY/K1. After partial *Pst*I and T4 DNA polymerase treatment, pAY/K1.1 through −1.3 were obtained, each lacking one of the three *Pst*I sites of pAY/K1. By fusing pAY/K1.2 and pAY/K1.3, plasmid pAY/K2 was constructed, carrying a unique *Pst*I site between the *amyE* segments. This site was subsequently used for the insertion of PCR fragments carrying either the *Pseudomonas putida xylE, E. coli lacZ*, or *Aequorea victoria gfp* gene flanked by *Pst*I and *Nsi*I sites, yielding pROBe1, pROBe4, and pROBe5, respectively. All these vectors contain a unique *Bgl*II site that was used for the introduction of *D. radiodurans* R1-derived promoter fragments that were amplified by PCR and cloned into pCR2.1 or pCR2.1-TOPO. A second series of vectors were constructed by deletion of an *Nhe*I-*Xba*I fragment from pROBe1 carrying the *amyE* (pAMYE1), *lexA* (pLEXA1), and *groESL* (pGROES1) promoter fragments and subsequent cloning of a pMTL23-derived multiple cloning site in a unique *Xma*III site located behind these promoter fragments. In a final step, the *E. coli lacZ* gene was inserted in the *Bgl*II and *Spe*I sites of these vectors, generating plasmids pAMYE4Z, etc.

Detection of α-amylase activity. Km^r *D. radiodurans* R1 transformants were streaked on TY agar (TGY from which glucose was omitted), supplemented with 1% (wt/vol) soluble potato starch (Sigma, S-2004). After growth at 30°C for 2 days, plates were placed at room temperature for an additional 5 days. Haloes around α-amylase-producing colonies were visualized using an iodine solution consisting of 0.6% (wt/vol) KI and 0.3% (wt/vol) I_2.

β-Galactosidase assays. Expression of the *lacZ* reporter gene in *E. coli* and *D. radiodurans* colonies was detected using X-Gal (40 μg/ml). Quantitative analyses of *lacZ* expression were performed according to Miller et al. (8). Cell extracts of *D. radiodurans* were obtained by passing concentrated cell suspensions through a French press at 1,000 lb/in^2 using a J5-598A laboratory pressure cell press (Aminco, Silver Spring, Md.). To prevent degradation of the reporter protein in cell extracts, a protease inhibitor cocktail (Complete Mini; Roche) was used. Alternatively, β-galactosidase activity was measured in toluene-permeabilized cells as follows. Cells were harvested by centrifuging 1-ml culture samples at 16,000 × *g* for 2 min. The pellets were subsequently resuspended in 500 μl of Z-buffer (8) supplemented with lysozyme (25 μg/ml) and DNase I (50 ng/ml). After incubation for 30 min at 37°C, 20 μl of toluene was added. The suspensions were incubated for another 60 min at 37°C, and aliquots (20 to 200 μl) were taken to measure β-galactosidase activity.

Northern hybridizations. Total RNA was isolated from a maximum of 5 optical density at 600 nm (OD_{600}) units of exponentially growing cells using the RNA Perfect kit (Eppendorf-5 Prime Inc., Boulder, Colo.). The isolates were subsequently treated with RNase I-free DNase I (Gibco-BRL) to remove residual contaminating chromosomal DNA. RNA samples (±8 μg) and molecular size RNA markers (0.24 to 9.5 kb; Gibco-BRL) were electrophoresed on MOPS (morpholine propane sulfonic acid)-formaldehyde-agarose slab gels at 6 to 7 V/cm. Subsequently, RNA was either stained in ethidium bromide (1 μg/ml) for 20 min and destained overnight in diethyl pyrocarbonate-treated H_2O or transferred to positively charged Hybond-N$^+$ membranes (Amersham Pharmacia Biotech) and probed with ^{32}P-labeled PCR fragments, according to Sambrook et al. (13).

Transcription start site mapping. Transcription start sites were mapped by means of primer extension according to the ThermoScript cDNA synthesis protocol, using 10 μg of total RNA. Primers were labeled with [γ^{32}P]-ATP (6,000 Ci/mmol; NEN) using T4 polynucleotide kinase (Roche). In each case, primers for the reverse transcription reactions were 18- and 30-mers and were located at different positions relative to the start codon.

RESULTS

Construction and characterization of an integration vector. In order to develop a system for analyzing promoters in *D. radiodurans* at the same copy number as the chromosome,

TABLE 2. Overview of primers used for cloning, diagnostic PCR, and primer extension experiments

Primer	Length (bp)	Sequence[a]	Chromosome or plasmid, position[a]
Cloning and diagnostic PCR			
*amyE*_fwd	20	CCCTGACATCCCGCCTCTGA	I, 1484016
*amyE*_rev	21	TCACCGCAAAACGAACGCCGC	I, 1485526 (C)
amyE-190	17	CACCAGAAGGCGACGAT	I, 1483885
*amyE*82	17	CGGTTGATGTAGGCGCA	I, 1484147 (C)
*amyE*229	18	TTCTGGATGTAAGGCAGC	I, 1484304 (C)
groESL-540	18	AATCACCGCGTACTCGTC	I, 617330
groESL-236	17	GGAAGCACGTATTGTCG	I, 617634
*groESL*61	17	GTCTTCTGCTCGGCTTC	I, 617931 (C)
lexA-241	17	GAGCGCTACGCCTTCAT	II, 380033 (C)
*lexA*46	17	GTGGCTTGCAGGATGGA	II, 379746
*polA*fwd	18	CAGAAGGTCCAGAACGTG	I, 1732780 (C)
*polA*_PE	18	AGGGATCTGGGGAAGCGT	I, 1732302
*rpoBC*fwd	18	GTCCTTGCGCATCGTCTG	I, 927583 (C)
*rpoBC*_PE	18	CTGCACTTCGGTCAGGTT	I, 926953
*rpoD*fwd	18	CTCCCAAAAAGGCCCGTG	I, 929104
*rpoD*_PE	18	GGTCCTGCACTTCGATGT	I, 929549 (C)
*amyE*DCOf	18	GAACAAGGACGTGGACTG	pAY/K2, 859
*amyE*DCOr	18	CCACGTTGTATACGGCTT	pAY/K2, 2726 (C)
Km^RDCO	18	ATCCTGGTATCGGTCTGC	pAY/K2, 1999 (C)
SCOfwd	18	GAACTGGATCTCAACAGC	pAY/K2, 3791
SCOrev	18	AGGCACCTATCTCAGCGA	pAY/K2, 4496 (C)
Primer extension			
*aceR*_PE	18	GCGTCAGAATCTCGGCAT	II, 300602
*aceR*_PE30	30	TACTCGGGCTTGATCGGCGCGTTGATGGTC	II, 300619
*amyE*_PE	18	ATCTGGCCTTCAAAGCTG	I, 1484169 (C)
*amyE*_PE30	30	ACCTGATAGATGATCTGGCCTTCAAAGCTG	I, 1484181
*lexA*_PE3	18	CTGATTGCCTGCTTGGTG	II, 379674
*lexA*_PE30	30	GTGATGCCCACTTCCTGCGCCACCTGCCCC	II, 379689
*polA*_PE	18	AGGGATCTGGGGAAGCGT	I, 1732302
*polA*_PE30	30	GGGAGGATTCTACTCTGGACGTAATTGCCG	I, 1732328
*rpoBC*_PE	18	CTGCACTTCGGTCAGGTT	I, 926953
*rpoBC*_PE30	30	GGTCAGGTTGGGGAGCGGAATCACTTCGGT	I, 926962
*rpoD*_PE	18	GGTCCTGCACTTCGATGT	I, 929549 (C)
*rpoD*_PE30	30	AGGTAGACCTGCATATCCTCGAAGGCGTCA	I, 929520 (C)

[a] I and II, chromosome I or II, respectively. (C), sequence is on noncoding strand.

a chromosomal integration vector was developed based on recombination replacement within a nonessential gene. A gene predicted to encode α-amylase was chosen as the target insertion site, as similar systems have proven successful in other bacteria (4, 6). Insertions in this gene, encoding the 1,4-α-D-glucan glucanohydrolase enzyme, provide a screenable phenotype, the formation of turbid haloes around amylase-producing colonies on starch-containing agar plates. Analysis of the *D. radiodurans* R1 partial genome sequence indicated that a 1,452-bp open reading frame (ORF) (DR1472) located on chromosome I was a likely candidate for an α-amylase ortholog, with identities to the *E. coli malS* and *B. subtilis amyE* genes. The predicted protein contains a possible signal peptidase cleavage site (^{17}AQA ↓ AP^{21}), suggesting that it may be translocated across the bacterial membrane. The corresponding ORF was amplified by PCR and cloned in pCR2.1. The pAY/K (general insertion vectors) and pROBe (insertion vectors containing reporter genes; Fig. 1) series of plasmids were subsequently constructed as indicated in Materials and Methods. Transformation of $CaCl_2$-competent *D. radiodurans* yielded Km colonies at low frequencies when the plasmids were propagated in *E. coli* JM109 (Table 3). Loss of function could be visualized by the absence of halos around colonies grown for several days on TY agar containing 1% (wt/vol) starch.

Using the pAY/K-derived series of plasmids, the use of linearized DNA greatly enhanced the occurrence of replacement recombinants (double-crossover strains) as opposed to single-crossover recombinants. However, the use of nonmethylated donor DNA, DNA passed through a *dam dcm E. coli* strain (11), resulted in large increases in transformation efficiencies (Table 3). Hence, it seemed possible that restriction of methylated donor DNA caused by a methylation-specific endonuclease(s) might significantly affect transformability of *D. radiodurans* and that by inactivating these systems, an improved host for genetic engineering of *D. radiodurans* might be obtained. Although a candidate (DRB0143) with identity to the *E. coli mcrBC* operon was identified in the partial genome sequence (12), an insertion mutation generated in *mcrB* did not cause increased transformation frequencies. All transformations with these and subsequent integration vectors were carried out after passage through a *dam dcm E. coli* strain.

In order to obtain *D. radiodurans* fragments containing promoter activity, two approaches were used with these vectors: direct cloning of PCR products based on the genome sequence and a random screening approach.

Assessment of promoter activities from selected genes. Analysis of the preliminary genome sequence generated a number of potentially interesting genes for promoter analysis. Three genes were chosen at this stage, *amyE* (since it was being used as an insertion site) and two that might be under stress

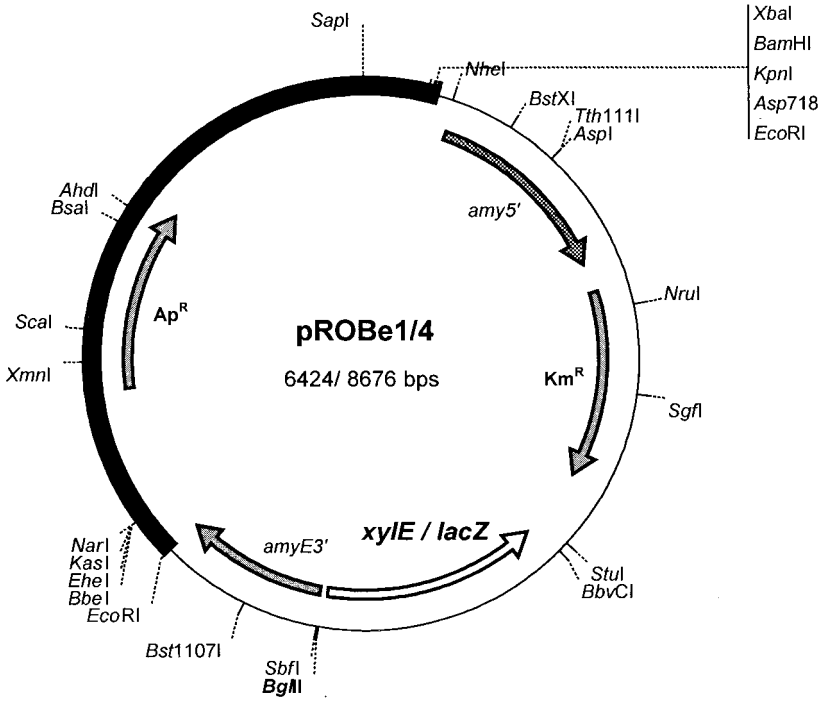

FIG. 1. Schematic representation of the integration vectors and their unique restriction sites. The checkered and solid gray bars represent the 5′ and 3′ portions of the *D. radiodurans* R1 *amyE* gene, respectively; the solid bar indicates the pUC19-derived vector moiety. Antibiotic resistance markers (Ap^r and Km^r) are represented as shaded arrows. Fragments to be fused to the reporter genes *xylE* and *lacZ* (open arrow) were inserted in the *Bgl*II site indicated in bold type. Also indicated are the *Eco*RI sites used in the generation of pAY/K1 (see text for details). The vectors that were used for the integration and analyses of the promoters described in the present studies were different in their orientation of the P_x::*lacZ* fusion (see Materials and Methods).

control, *groES* (DRO606), and *lexA* (DRA0344) (5, 16). The latter two were chosen as candidates for future development of stress-induced expression systems. In *D. radiodurans* (as in most other bacteria), *groES* is located immediately upstream of *groEL* (DRO607) (17), and both are involved in heat shock response in other bacteria (5). Using the vectors described above, transcriptional fusions between putative promoter fragments and reporter genes present on pAY/K2 plasmids were constructed to assess the activity of these promoters. The fragment containing the *groES* regulatory region was chosen so as to include a possible σ^{32}-like promoter sequence (15) located 157 bp upstream of the *groES* translational start site. For *lexA* and *amyE*, each promoter fragment tested included approximately 200 bp upstream of the predicted start codon. Two of the reporter genes tested (*xylE* and *lacZ*) were reliable for plate screening, but only *lacZ* gave detectable activity in in vitro assays. Therefore, *lacZ* was used as a reporter in experiments involving activity measurements.

When these promoter fragments were fused to *lacZ*, blue colonies of *D. radiodurans* were obtained on X-Gal plates and significant levels of β-galactosidase activity were detected in cell extracts of these *D. radiodurans* recombinants (Table 4). The *lexA* fragment showed relatively low activity, the *amyE* fragment had moderate activity, and the *groES* fragment showed high activity (Table 4). Similar levels were obtained with a more rapid procedure involving toluene-treated cells (see Materials and Methods). The double-crossover nature of these recombinants was verified by a series of diagnostic PCRs on chromosomal DNA isolated from these strains.

The *groES* promoter appeared to be expressed at a high, constitutive level at the normal growth temperature (30°C). In

TABLE 3. Efficiencies of *D. radiodurans* R1 transformation[a]

Plasmid	Strain	Transformation frequency (transformants/μg of DNA)	Ratio, GM48/JM109
pAY/K2	JM109	14	47.6
	GM48	667	
pROBe4	JM109	1.6	751
	GM48	1,202	

[a] Transformation yields with DNA isolated from a *dam dcm* strain of *E. coli* (GM48) were compared to those obtained with DNA extracted from a methylation-proficient host (JM109).

TABLE 4. Assessment of promoter activities in *D. radiodurans* R1 strains containing double-crossover promoter fusion insertions using the *E. coli lacZ* reporter gene

Strain	Relevant genotype	β-Galactosidase activity (nmol/min/mg of protein)
R1[a]	Wild type	0
RM11	Δ*amyE* P*amyE*::*lacZ*	47.5
RM15	Δ*amyE* P*lexA*::*lacZ*	9.2
RM16	Δ*amyE* P*groES*::*lacZ*	115.1
RM18	Δ*amyE lacZ* (no promoter)	0.9

[a] *D. radiodurans* R1 wild-type culture was used as a zero reference in the β-galactosidase assay.

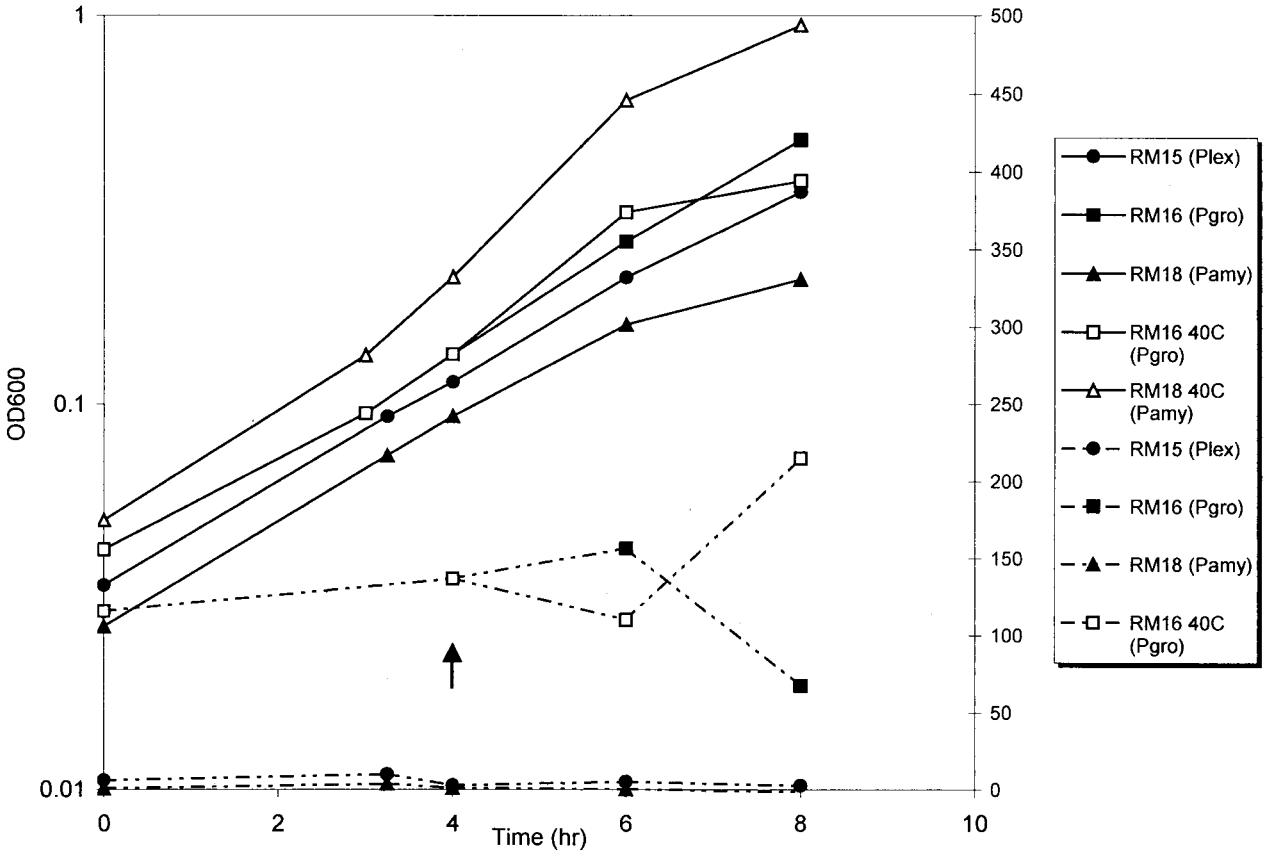

FIG. 2. Growth (solid lines) and β-galactosidase expression (dotted lines; nanomoles per minutes per OD_{600}) of strains RM15 (Δ*amyE* P*lexA*::*lacZ*), RM16 (Δ*amyE* P*groESL*::*lacZ*), and RM18 (Δ*amyE*::*lacZ*). The time point at which cultures were shifted to elevated temperatures is indicated by the arrow.

many bacteria, these genes are expressed at higher levels under heat shock conditions (5). In order to assess whether the cloned promoter region contained a heat shock regulatory element, we analyzed expression of the *groES* promoter under heat shock conditions. For *D. radiodurans* in TGY medium, the nonpermissive temperature is 42°C (7a), and so 40°C was chosen for the heat shock temperature. Four hours after a shift from 30 to 40°C, growth had leveled off, but a threefold increase in activity was observed compared to a culture with no temperature shift (Fig. 2). A similar difference was observed in cells grown overnight at 40°C compared to cells grown at 30°C.

Random screening for promoter fragments. In a separate series of experiments, a random *Sau*3A genomic library of *D. radiodurans* R1 was constructed in pROBe1 in order to isolate promoter-containing fragments from the genome. After establishment of recombinants in *E. coli*, clones were pooled, transferred to *D. radiodurans* R1, and screened for catechol 2,3-dioxygenase activity on plates. Pools generating transformants showing activity were separated into single clones which were tested again for activity in *D. radiodurans* R1. Several promoter-containing fragments were identified by this approach. A few showing the strongest activity were sequenced, and among these was a fragment derived from a gene that shows considerable similarity with the malate synthase A gene (*aceB*) of *E. coli*. By analogy, we designated the corresponding ORF *aceR*. The 5′ terminus of this gene is identical to an ORF (DRA0277) described by White et al. (17). This putative promoter fragment was included in further studies.

Northern blots and transcriptional start site mapping. In order to further characterize expression of these genes, Northern blots were carried out. Total RNA was isolated from cells at different points in the growth cycle (Fig. 3A). Subsequent Northern hybridization experiments identified transcripts for *amyE* and *groESL* (1.45 and 2.02 kb, respectively) with RNA from early-exponential-phase cells, but we were unable to detect a *lexA* transcript in any of the RNA preparations (Fig. 3B). For *amyE* and *groESL*, the mRNAs detected were the correct size for single-gene and two-gene transcripts, respectively.

Transcription start sites were successfully mapped for *groESL, aceR*, and *lexA* (Table 5), but not for *amyE*. It is not clear why we were unsuccessful with the *amyE* promoter, since the Northern blots suggested the presence of a detectable amount of transcript, but several attempts were made with different RNA preparations and different primers and all were unsuccessful. For *groESL*, a major and a minor band were identified (Fig. 4). Transcription start site mapping experiments for this region were carried out with RNA isolated from cultures grown at 30 and 40°C, and in both cases the same transcription start sites were obtained as major and minor bands. Neither of these corresponds to the σ^{32}-like promoter sequence found in this region, which overlaps but does not coincide with the minor start site (Table 5).

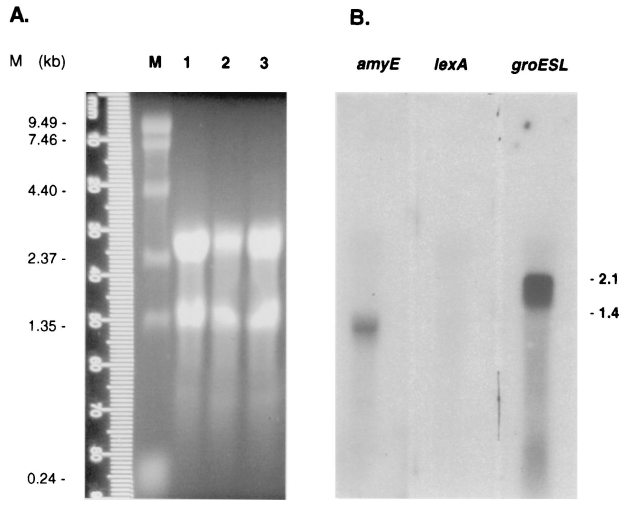

FIG. 3. RNA analyses in wild-type cells of *D. radiodurans* R1. (A) Formaldehyde gel electrophoresis of total RNA isolated from stationary-phase (lane 1), early-exponential-phase (lane 2), and mid-exponential-phase (lane 3) cells. As a marker, 4 μg of an RNA ladder was applied (lane M). (B) Northern hybridization using probes for *amyE, lexA*, and *groESL*. The expected transcript sizes are indicated at the right-hand side of the panel (in kilobases).

In order to obtain additional start sites and promoter sequences, we selected other genes that were expected to be expressed at detectable levels in exponentially growing cells. For this purpose, the *rpoBC* (DR0912 to DR0911) genes were chosen, predicted to encode subunits of RNA polymerase (17), and two plasmid-encoded genes in pI3. pI3 is a derivative of a large plasmid found in *D. radiodurans* SARK that also replicates in *D. radiodurans* R1 (7), and we have sequenced the *Deinococcus* insert in pI3 in its entirety (7a). Two plasmid genes were chosen that appeared to be expressed at high levels: *resU*, encoding a putative resolvase, and the antibiotic selection marker *cat*. A putative promoter segment for *rpoBC* was amplified by PCR, and the start site was mapped by primer extension (Table 5). The start sites for the pI3 promoters were mapped directly from pI3 using RNA from a *D. radiodurans* R1 strain containing pI3. Surprisingly, all seven of the upstream −10 and −35 regions bear a resemblance to the standard *E. coli* σ^{70} promoter sequence. The *rpoBC* and *groESL* promoters show only one and two differences, respectively, from the σ^{70} consensus sequence, in both cases in the −10 sequence.

DISCUSSION

Despite the fact that *D. radiodurans* R1 has been the focus of increasing scientific interest over the past decade, only a limited number of molecular tools for genetic engineering of this radioresistant bacterium are currently available and very little is known about promoters and expression. The present paper describes the development of a series of double-crossover integrative vectors and their use in the cloning and initial characterization of promoters isolated from the *D. radiodurans* R1 genome and from a cryptic *D. radiodurans* SARK plasmid.

Although these integrative vectors were used in this study for promoter cloning, they are also useful for general cloning and expression in this strain. These vectors, in combination with the improved transformation protocols described here, will significantly enhance the ability to carry out genetic manipulations in *Deinococcus* strains.

Of the three potential reporter genes tested, only *lacZ* was successful for screening of colonies as well as for quantitative assays in cell lysates and in toluene-permeabilized cells. This system was used to identify three promoters with different strengths, one low level (*lexA*), one moderate level (*amyE*), and one strong (*groESL*). It was surprising to find a *lexA* ortholog in the *D. radiodurans* R1 genome sequence, because this strain was reported not to have an SOS-like error-prone response to DNA damage (10). Low-level reporter activity was obtained with the fragment tested, but the role of *lexA* in *D. radiodurans* R1 remains unclear.

Transcriptional start sites were mapped for five genes, including *groESL*. Alignment of the −10 and −35 regions of these sequences showed that two strong promoters (*groESL* and *rpoBC*) had −10 and −35 regions that were highly similar to the corresponding regions of the *E. coli* σ^{70} consensus promoter. This result was surprising because previous reports had suggested that *Deinococcus* promoters should be significantly different from *E. coli* promoters (7, 14). The other three regions upstream of mapped transcriptional start sites as well as an additional minor start site for *groESL* were more divergent, but the −10 and −35 regions still showed some similarity to the *E. coli* consensus sequence.

The two start sites mapped for *groESL* were about 60 bp

TABLE 5. Alignment of transcription start sites of *D. radiodurans* R1 genes[a]

Promoter	−35 and −10 sequence, +1 nucleotide, and spacing (bp)	Position relative to RBS and ATG
groESL$_M$	TTGACATTTTTCTTATCGGCGCTCTACCATCCGTGA* (18)	N_{66}-**AGGAGGA**CCCCAC*ATG*
groESL$_m$	GTCCCAGCGCCCCTTGAGCGTCATAGACTCAGA* (17)	N_{120}-**AGGAGGA**CCCCAC*ATG*
aceR	GGAACAGCCGGCCTCATTTGCTGTAAAATGAAATC* (17)	N_{57}-**AGGAGAA**CCCC*ATG*
lexA	TCCTCGTAGGCCAATTCTGACGGTTGGGCCGCCGCTTG* (17)	N_{18}-**GGCAAA**CTGCGCGCAC*ATG*
pI3 *resU*	GGAACCCCGCGCCGACCCCCCTTAATGCGGC* (16)	N_{119}-**AGGAGG**TTTGA*ATG*
pI3 Cm^r$_M$	GGTCCTCGCCCTCCTTAGGGGCAAGGACGTCCGGC* (18)	N??-??
rpoBC	TTGACAGGGAATCATGAGCGCCCTATACTTTC* (17)	N_{168}-**GAGG**TGTGC*ATG*
E. coli σ^{70}	TTGACA-N_{17}-TATAAT	

[a] The nucleotides at which transcription is initiated (+1) are marked with the asterisk (*); −35 and −10 sequences are underlined. Start codons are in italics, and the putative ribosome-binding site (RBS) is in bold. Since the region immediately downstream of the transcriptional start site of the promoter driving expression of the Cm^r marker was lost during the construction of PI3, the original gene and start codon are not known; this is indicated with question marks. Major (*groESL*$_M$) and (*groESL*$_m$) minor start sites are shown, as well as the putative σ^{32} promoter sequence preceding the *groESL* operon (double underlined).

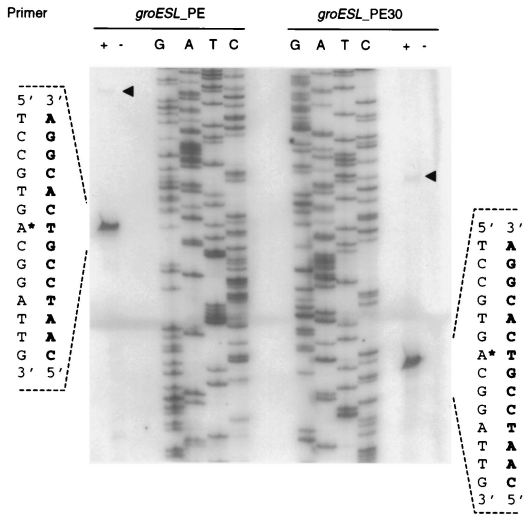

FIG. 4. Transcription start site mapping of the *groESL* promoter by primer extension. The experiments were performed in either the presence (+) or absence (−) of reverse transcriptase, and the two panels show the results with two different primers (PE and PE30). The flanking sequences represent the upper (lightface) and lower (boldface) this is the strand shown) strands. The nucleotide used as the transcription initiation site is marked with an asterisk. An additional minor cDNA product (◀) was obtained in both reactions; these could reflect an alternative transcription initiation site.

apart. The minor (more upstream) start site overlapped a sequence with good similarity to the *E. coli* heat shock sigma factor (*rpoH*) recognition consensus sequence, which is involved in regulation of heat shock genes such as *groESL* in many bacteria (5). However, the −10 and −35 sequences upstream of this minor start site did not coincide with the same regions in the *rpoH* recognition sequence. The possibility that the transcription start site might be different under heat shock conditions was addressed. Although the cloned *groESL* promoter region was found to direct higher expression of the reporter gene under heat shock conditions, suggesting that *D. radiodurans* mounts a heat shock response, the transcriptional start sites were the same as in cells grown at normal temperatures. The basis for heat shock regulation in *D. radiodurans* is unknown, and further studies will be required to address this.

The present studies show that it is possible to efficiently insert heterologous genes into the genome of *D. radiodurans* using the vectors described in this paper. In addition, the constructs containing the various promoters described here can be used for insertional expression vectors with different levels of expression. Such new genetic tools will greatly enhance the ability to carry out genetic manipulations of *D. radiodurans* for a variety of basic and applied research applications.

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